Studying X chromosome inactivation in the single cell genomics era

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Abstract
Single cell genomics is set to revolutionise our understanding of how epigenetic silencing works; by studying specific epigenetic marks or chromatin conformations in single cells, it is possible to ask whether they cause transcriptional silencing or are instead a consequence of the silent state. Here we review what single cell genomics has revealed about X chromosome inactivation, perhaps the best characterised mammalian epigenetic process, highlighting the novel findings and important differences between mouse and human X inactivation uncovered through these studies. We consider what fundamental questions these techniques are set to answer in coming years and propose that X chromosome inactivation is an ideal model to study gene silencing by single cell genomics as technical limitations are minimised through the co-analysis of hundreds of genes.

Abbreviations
NGS, Next Generation Sequencing; scRNA-seq, single cell RNA sequencing; XCI, X chromosome inactivation; Xa, active X; Xi inactive X; mESC, mouse embryonic stem cells; histone H3 lysine 4 trimethylation, H3K4me3; Xm, maternal X; Xp, paternal X; Xic, X inactivation centre; ZGA, zygotic genome activation; BS-seq, bisulphite sequencing; COOL-seq, Chromatin Overall Omic-scale Landscape Sequencing; NOMe-seq, Nucleosome occupancy and DNA methylation sequencing; HiC, genome-wide chromosome conformation capture; PBAT-seq, post bisilfute adaptor tagging sequencing; NMT-seq, NOMe-seq plus transcriptome sequencing

Introduction
Our understanding of life and the complexities of the biological processes that underlie it are tightly linked to the technology of the time. This truth has perhaps never been more apparent than with the development of Next Generation Sequencing (NGS) technology and our entry into the Genomic Era slightly more than a decade ago. NGS has allowed us to probe the genome in more detail than ever before, in a rapid, affordable and accessible manner. Both the quantity of data being produced, and our biological understanding are growing at a pace never seen before. However, a significant caveat to what we have learned from genomics is that due to the large amount of genomic material required to perform the techniques, we are necessarily assaying the contents of a pool of cells, thus gaining average measurements across cell populations. As a consequence, we are potentially missing important heterogeneity between cells (Figure 1). Recently, technology has emerged to redress this issue with modifications to existing genomics approaches that allow for sample inputs of just a single cell.

Studying cell populations at the single cell level is not new, with techniques such as microscopy and Fluorescence Activated Cell Sorting (FACS) being applied with great success for many decades, and likely for many more to come; however, the arrival of single cell genomics promises a transformative change in our ability to understand biology at the level of the single cell. The first single cell genomics technique arrived in 2009 with the publication of single cell RNA sequencing (scRNA-seq) [1] and has become by far the most adopted of the single cell sequencing methodologies. scRNA-seq has been applied to a multitude of questions across many disciplines of biology resulting in key discoveries, including heterogeneity of transcription within cell types [2-4] and the identification of novel cell subtypes [5-7]. Moreover scRNA-seq has allowed detailed transcriptional analysis of rare and hard to obtain cell types, particularly cells of the human pre-implantation embryo [8]. Now single cell genomic techniques exist for sequencing of genomic DNA [9], detection of the epigenome [10-12], mapping of modified histones [13] and chromatin structure [14-18]. More recently methods have been developed that combine single cell genomic technologies to facilitate the detection of the genomic DNA, transcriptome and epigenome from within a single cell [19-24]. This exciting advancement, known as multi-omics, will elevate the power of single cell sequencing from profiling of cellular features to understanding the intricate mechanisms of gene regulation and how this contributes to cell fate decisions in both normal development and disease. There are significant technical challenges to be considered before embarking on a single cell experiment and indeed when analysing the results. Some of these we discuss below and have also been documented in a number of excellent reviews [25-28].

scRNA-seq has already been applied to further our understanding of X chromosome inactivation (XCI). This review details the current progress in applying single cell sequencing to the study of XCI and how these technologies may be applied in the future to answer outstanding questions in the field. We focus on XCI since it is a powerful model, which provides information on how epigenetic silencing occurs chromosome-wide.

XCI: Basic concepts and outstanding questions

In diploid mammals, the inherent X-linked dosage imbalance between XX females and XY males is corrected by the almost complete silencing of a single female X chromosome [29]. This process, known as XCI, represents the best characterised epigenetic silencing events. Because the silencing of hundreds of genes can be studied in parallel, XCI provides an excellent system in which to study epigenetic repression more broadly, particularly through the application of genomics. Most of what we know about XCI has been learned by studying the mouse as a model system and this introduction will focus solely on the mouse; however, recent studies utilising scRNA-seq report that human XCI differs in some fundamental ways and this will be discussed in detail later in the review (Figure 2).

In vivo, XCI occurs in different variations in the murine pre-implantation and post-implantation embryo [30]. The four to eight cell embryo displays an imprinted form of XCI, where the paternal X chromosome is selectively silenced [31]. Imprinted XCI persists in extraembryonic tissues throughout development, but the silenced paternal X chromosome undergoes reactivation in the embryonic inner cell mass of the blastocyst, and for a brief developmental period these cells carry two active X chromosomes (Xa)[32-35]. The second variation of XCI occurs in the epiblast of the

pre-implantation embryo and results in the seemingly random silencing of either the maternal or paternal X chromosome and once established this silencing is stable through mitosis with all future daughter cells inheriting the same inactive X chromosome (Xi) as the parent cell [36, 37]. *In vitro*, mouse embryonic stem cells (mESCs) retain the XaXa status of the inner cell mass from which they are derived, but upon differentiation in culture these cells undergo random XCI [38, 39].

XCI is initiated by induction of the *Xist* noncoding RNA that spreads to coat the future Xi in *cis* [40, 41], leading to exclusion of RNA PolII [42]. *Xist* silencing depends on its binding partner Spen [43-45] and rapidly results in loss of active histone marks including acetylation [46] and histone H3 lysine 4 trimethylation (H3K4me3) [47] and acquisition of the repressive histone marks H2AK119ub1 [48-51], H3K27me3 [52-54] and H3K9me2/3 [47, 55-58]. The Xi localises to the silent territory at the nuclear periphery and adopts a distinct bipartite structure in three-dimensional space that lacks the more complex three-dimensional organisation typical of active chromosomes [59-61]. DNA hypermethylation of CpG islands (CGIs) on the Xi occurs late in the ontogeny of XCI and is required to maintain the silent state [46, 62] along with recruitment of Smchd1 [63, 64] and Setdb1-mediated H3K9me3 [58, 65].

The above details only the major events of this well characterised process, however there are several open questions that the application of single cell sequencing could shed light upon. Several recent publications have employed scRNA-seq to study XCI and have begun to answer some of these questions. For the remainder of this review we will discuss the progress made by these publications and what could further be achieved with single cell technology.

Lessons from scRNA-seq in mouse cells

To date, the publications that offer insight into mouse XCI from single cell sequencing segregate broadly into two groups: those that investigate the pre-implantation mouse embryo and therefore shed light on imprinted XCI; and those that study the early gastrulation embryo and inform on random X inactivation. Pleasingly, these studies tend to confirm previously established principles of XCI, still, some intricacies of the process are beginning to emerge. As with other single cell studies of cell populations, scRNA-seq has revealed heterogeneity in XCI between equivalent cells during mouse embryonic development both *in vivo* [66, 67] and *in vitro* [68] (Figure 1). X reactivation was found to begin as early as embryonic day (E) E3.5 [67], however reactivation was not complete in all cells until E5.5. Inactivation was equally heterogeneous with XCI being complete in some cells by E5.5, but still incomplete in others at E6.5 or even E7.5 [66]. It remains unclear whether this heterogeneity is due to a delay in development or in XCI itself, and to answer this question it will be necessary to develop robust methods to accurately measure developmental timing of single cells, possibly through transcriptomic staging. Another important consideration when determining heterogeneity from scRNA-seq data are the technical limitations of the technique. Due to the very low input of material, scRNA-seq data suffers from stochastic drop-out of transcripts, which may be misinterpreted as biological transcriptional heterogeneity. Moreover, as scRNA-seq captures the transcriptome of a cell at a specific time-point, biological phenomena such as transcriptional bursting or allelic asynchrony of transcription will also confound predictions of heterogeneity [69].

Interestingly, imprinted XCI appears less heterogeneous [2, 70] with the majority of cells showing complete XCI by the 16-cell stage persisting to the E3 blastocyst [67]. This difference in heterogeneity between random and imprinted XCI may simply reflect a more homogeneous starting point in the germ cell chromatin or perhaps the limited cell numbers that exist at this early developmental time. Alternatively, the mechanisms that establish imprinted XCI may be more tightly co-regulated between cells resulting in more uniform and complete gene silencing. A fundamental question for the field now is what, if any, impact does cellular heterogeneity of random XCI have on the developing embryo and does this reflect developmentally rare cell populations of the pre- and post- implantation embryo?

scRNA-seq experiments are also beginning to shed light on the more mechanistic features of XCI. It has been controversial whether imprinted XCI is established *de novo* or whether a pre-inactivated Xp is inherited and then propagated [33-35, 71]. However, scRNA-seq of the pre-implantation embryo with allelic discrimination shows clearly that both the Xm and Xp undergo zygotic genome activation prior to inactivation of the Xp, suggesting that imprinted XCI is indeed established *de novo*. So far genetic studies and now even single preimplantation embryo genetic studies have been informative in revealing some of the differences between imprinted and random XCI [72-74]. It will be interesting to extend these and similar studies to single cell genomics studies, to determine the precise differences in the requirements of imprinted and random XCI. scRNA-seq data from an embryo carrying an *Xist* deletion on the Xp demonstrated that *Xist* is required for the initiation of imprinted XCI, and would suggest that at least the requirement for *Xist* is shared between random and imprinted XCI [70].

Silencing on the X chromosome is thought to start and then spread out from the *Xist* locus, known as the X inactivation centre (Xic) [75, 76]. Single cell data has been able to resolve that *in vivo* imprinted XCI [2, 70] and random XCI *in vitro* [66] spreads out from the Xic (Figure 1). Still, in at least one of these reports the spreading was not completely linear [2] and another suggested that spreading occurred from gene-rich to gene-poor regions [66], implying spreading is a more intricate process. More recently there was found to be a seemingly ordered timing of reactivation of X-linked genes in the blastocyst as well [77]. Although not yet available, a multi-omics approach that allows the simultaneous detection of both the transcriptome and the higher order chromatin state within a single cell will reveal what role three-dimensional architecture plays in the spreading of X chromosome silencing. Additionally, striking differences in XCI timing were observed between mouse strains, suggesting mechanistic differences in how XCI is established exist between genotypes [19, 70]. Both studies also found chromosome specific escape genes, thereby suggesting not only heterogeneity in the establishment, but also the functional result of XCI.

Based on studies from 2008-2011, it was purported that XCI status is directly linked to the pluripotency network due to an anti-correlation between XCI and pluripotency factors in bulk expression analysis [78, 79]. Further, Pou5f1, Sox2 and Nanog binding sites in *Xist* intron 1 were linked to its regulation [78, 79], suggesting that loss of pluripotency gene expression is functionally required for the induction of XCI. In contrast to this however, the increased

resolution afforded by scRNA-seq has shown that in differentiating mESCs, although initiation of random XCI was globally correlated with loss of pluripotency factor expression, this was not the case in all individual cells [68]. Similarly, in another single cell transcriptomic publication, although Pou5f1 expression was found to correlate with X reactivation it was negatively correlated during XCI, and Nanog and Sox2 showed no correlation with XCI at all [67]. Together, these observations suggest XCI and pluripotency factors may not be functionally linked. Another publication offers yet another perspective on the relationship between XCI and pluripotency, suggesting two active X chromosomes stabilise the pluripotent state by inhibiting the MAPK and Gsk3 signaling pathways, resulting in maintained pluripotency gene expression and slower differentiation kinetics when compared to both XO and XY karyotypes [80]. Currently, the more critical experiments support this third model, however a functional analysis with a single cell read out may be required to finally resolve the issue.

scRNA-seq in mouse has provided a number of insights into the mechanism of XCI, yet it remains to be seen whether new factors involved in XCI will be validated by this approach. Intriguingly, two papers report the expression of a number of transcription factors that correlate with XCI in single differentiating mESCs [68] and single cells from the post gastrulation embryo [67]. Perhaps most excitingly is the correlation of both Dnmt3a and Zfp57 [67], as they have been shown to interact to maintain DNA methylation at imprinting control regions [81, 82] and Dnmt3a is thought to regulate *Xist* [83, 84].

Lessons from scRNA-seq in human cells

The ability to profile the transcriptome of a single cell offers a number of unique advantages that have opened the study of human XCI *in vivo*. Primarily, the very low input required for scRNA-seq makes it feasible to assay rare or hard to obtain cell populations, such as the human embryo. Secondly, the random nature of XCI means it is not possible to discern monoallelic expression in bulk RNA-seq data, as reads derive from multiple cells where either the Xm or Xp may be inactivated. This problem is typically overcome in mouse by skewing XCI through genetic deletion of *Xist* or making use of skewing that occurs between distantly related mouse strains; however, these solutions are not available in humans, making the ability to obtain the transcriptome of a single cell particularly useful to the study of random XCI in human. Accordingly, the first efforts to address XCI in the human pre-implantation embryo were reported in 2016, and excitingly suggested that human mechanisms differ substantially to that of the mouse [8] (Figure 2). As expected from previous studies [85-87], no evidence was found for the existence of imprinted XCI in human, but rather an alternative mechanism of dosage compensation was discovered by which expression from both active X chromosomes was partially reduced, in a process termed dampening. Dampening was not only observed in cells of the trophectoderm, where imprinted XCI occurs in mouse, but also in cells derived from the primitive endoderm and epiblast. Following zygotic genome activation by embryonic day 4, expression from the female X chromosome was approximately 1.8x that of the male. Dampening was established from embryonic day 5 and increased in effect until the study ended at embryonic day 7, where X-linked gene expression in the female was reduced to about 1.3x that of the male. Such a mechanism is exciting as it does not occur in mouse, although is a feature of sex chromosome dosage compensation in *C. elegans* [88], yet dampening in human female embryos remains controversial. Two recent publications

have offered a reanalysis of the Petropolous et al. data that employ stricter thresholds to characterise biallelic expression and find no evidence of dampening, but instead that transcriptional output from the X is reduced due a transition to monoallelic gene expression, indicative of XCI [89, 90]. As an obligate Xi is not genetically forced in these human data, as it might be in mouse models, it will be crucial to determine if the monoallelic expression observed here is indeed due to XCI and not allelic asynchrony. Dampening was also observed in naïve human pluripotent stem cells in culture [91], so although controversial the possibility of Xa dampening and the mechanisms that control it, merit further research. Of particular interest will be how female cells transition from dampening to random XCI and the factors that control this. Interestingly *XIST* may have a role in dampening as, unlike mouse, *XIST* is expressed from the Xa in the epiblast when the X chromosome is biallelic but dampened [8]. Moreover, during *in vitro* reprogramming of primed human pluripotent stem cells to the naïve state, *XIST* is initially expressed during random XCI and lost when the X chromosome is reactivated, however *XIST* expression returns in naïve cells where dampening occurs [91], so a role for *XIST* in the process is conceivable.

scRNA-seq is also revealing significant levels of escape from X inactivation in human cells (Figure 1). A large study that analysed the transcriptomes of 940 immune cells from 4 different females found that only 78% of measurable X-linked genes were uniformly inactivated in all samples, with the remaining genes variably escaping XCI [92]. Interestingly, the escaping genes are heterogeneous between identical cell types from different individuals as well as different cell types from the same individual. Moreover, escape genes were typically only expressed at 33% the level of their Xa counterparts, raising interesting questions about how much heterogeneity is tolerable in human XCI and perhaps gene dosage more generally, and how the epigenetic landscape of the X changed to allow expression of escape genes from an otherwise inactivated chromosome? Excitingly, high levels of escape from XCI suggests that inactivated alleles might be artificially reactivated, presenting an exciting opportunity for gene therapy where diseases are caused by heterozygous mutation of X-linked genes, such as Rett syndrome.

Single cell epigenomics and the future of single cell XCI research

Single cell multi-omics promises a lot, however the techniques are technically challenging, primarily due to having only a single DNA molecule from which to amplify within a cell, rather than potentially many RNA molecules for each gene available for scRNA-seq. Accordingly, there are some significant technical limitations that must be acknowledged prior to undertaking any single cell epigenomic technique. Primary for consideration is the fact that the entire genome is never captured due to stochastic technical drop out, thereby limiting the ability to truly assess presence or absence of an epigenetic mark. This is of particular concern for peak-based techniques such as DNaseI hypersensitivity, ATAC-seq and ChIP-seq that rely on the presence or absence of a read for data analysis. Techniques where features within the read provide the information, as opposed to the read itself, such as BS-seq and NOMe-seq, allow easy identification of regions where data is missing and therefore allow all reads to become informative. There are also a number of computational approaches that aim to tackle technical drop out including averaging over genomic bins [11, 12, 93], average over multiple cells [94] and applying machine learning [95], although there are also caveats to these approaches. Currently,

BS-seq and NOMe-seq, or multi-omics approaches that combine both (scNMT-seq and scCOOL-seq) appear to represent the most profitable single cell epigenomics techniques, however they also suffer with a large degree of technical noise, low mapping rates and high numbers of PCR duplicates.

Despite the current technical limitations, as XCI is a largely epigenetic process this is a necessary research area for the field and one that warrants an investment in technology development. Single cell epigenomic data pertaining to XCI is currently sparse with only a single paper addressing this question [19]. Using a novel multi-omics technique known as single cell COOL-seq that combines NOMe-seq with post bisulfite adapter tagging [96], Guo and colleagues were able to profile DNA methylation and nucleosome occupancy with allele specificity in the same cells from mouse pre-implantation embryos, finding higher levels of DNA methylation on the paternal X chromosome even after the effects of more rapid DNA demethylation of the paternal pronucleus were no longer apparent on autosomes, suggestive of imprinted XCI. This proof of concept study lacked the cell numbers required for statistical power; however, there was clearly heterogeneity in the DNA methylation and nucleosome positioning of the imprinted Xi. Unfortunately, COOL-seq does not currently allow RNA-seq from the same cell, but this would presumably be possible by separating the nucleus for scCOOL-seq from the cytoplasm for RNA-seq, as has been done for other multi-omics techniques [24, 97]. This would allow interrogation of how heterogeneity in epigenetic marks contributes to heterogeneity in XCI, with particular interest for escape genes. It will be exciting to see how much heterogeneity in the epigenome can be tolerated while still allowing effective gene silencing.

Several multi-omics approaches have been reported that allow the gathering of epigenomic and transcriptomic information from the same single cell (Table 1), see these reviews for what is available [25, 26, 28]. Currently, none of these approaches have been applied to the study of XCI, however they have the power to address some fascinating questions surrounding XCI and indeed epigenetic gene silencing more generally. For example, what repressive epigenetic marks, if any, are deposited prior to gene silencing and can we attribute causality to any of these marks (Figure 1)? Are there intricate differences in both the spreading of repressive marks and silencing of genes during the establishment of XCI? These questions are answerable by single cell multi-omics and are of fundamental importance in defining epigenetic marks as either the effectors or merely the consequence of gene silencing, both of the X chromosome and genome wide.

Many of the XCI discoveries made using transcriptomics have been validated in single cells by fluorescence *in situ* hybridisation (FISH), however single cell epigenomics will allow for single cell resolution of chromatin accessibility and 3-dimensional nuclear architecture, which are less easily assayed by traditional microscopy techniques. An interesting feature of the Xi is that it forms a bipartite structure of two seemingly unstructured mega domains, though these domains may indeed be structured but appear unstructured in bulk analysis due to extreme heterogeneity (Figure 1). Single cell genome-wide chromosome conformation capture (Hi-C) has been employed to study the male active X chromosome, however allele specific single cell Hi-C in female cells would be required answer this question [14]. Indeed, single cell resolution Hi-C may enable a

clearer understanding of how nuclear folding relates to gene expression generally, in what is often a hard to interpret field.

Concluding remarks

In their essence, many of the key questions surrounding XCI are also fundamental to epigenetic gene silencing more broadly. It remains uncertain what the functions of the various epigenetic marks are and what is the order of their establishment in the formation of a repressive chromatin state and gene silencing. Multi-omics approaches that deliver transcriptomic and epigenomic data from the same cell allow the single cell field to move beyond profiling heterogeneity and towards answering mechanistic questions pertaining to XCI and epigenetic silencing in general. Multi-omics approaches are still in their infancy and suffer from the limitation of technical dropout more so than the individual single cell genomics techniques, however as it involves the silencing of hundreds of genes in parallel the study of XCI has the potential to overcome this issue and reveal new mechanistic features of epigenetic gene silencing, just as the field has done for many years previously.

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Figure legends

Figure 1. Alternative conclusions from single cell and bulk cell analysis for some key facets of XCI

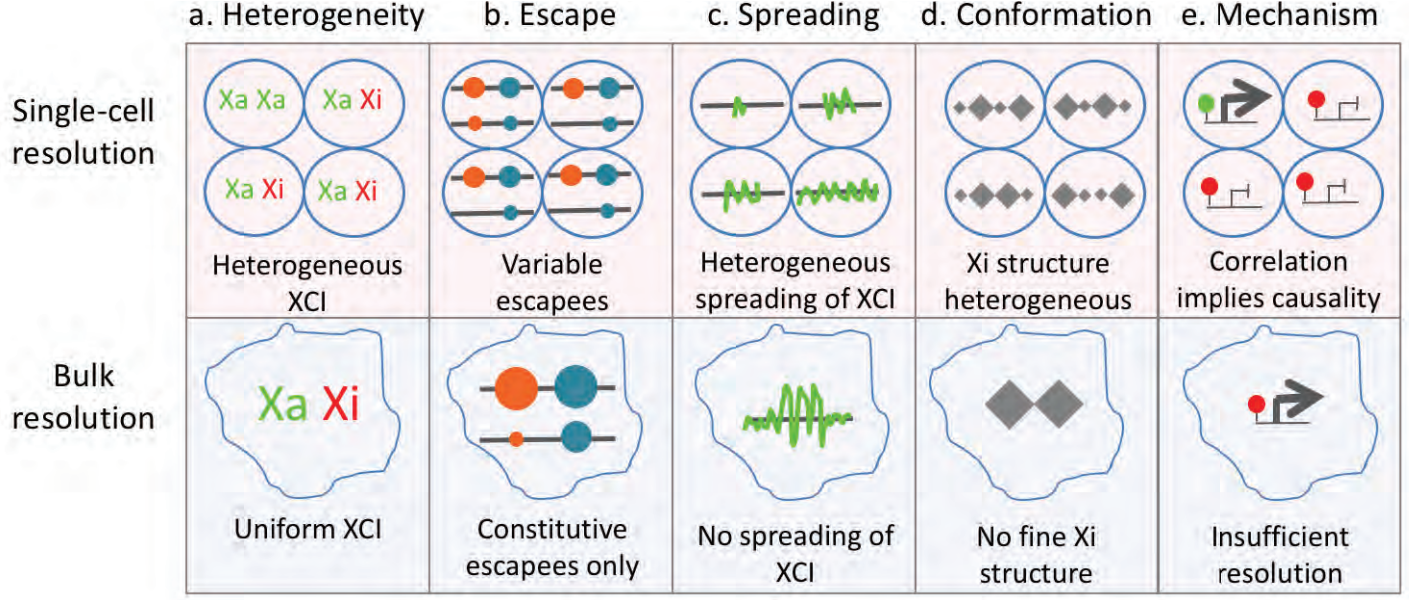
a. Single cell resolution allows the detection of heterogeneity in XCI between cells. The Xa and Xi are indicated. **b.** Single cell resolution allows the detection of variable escape genes that are below detection limits in bulk analysis. Variable (orange dots) and constitutive (blue dots) escape genes are indicated. **c.** Single cell resolution reveals spreading, both of epigenetic marks and gene silencing, whereas bulk analysis shows only the overall trend. The green line depicts the spread of silencing. **d.** Single cell resolution might reveal structured heterogeneity in the conformation of the Xi, whereas bulk analysis suggests lack of structure within bipartite lobes. Chromosome conformation of the Xi is depicted as grey triangles. **e.** The order of deposition of epigenetic marks to gene silencing in single cells will help define these marks as either the cause or consequence of silencing, however this relationship is masked in bulk cell analysis. Active genes (arrows), silent genes (bars), active epigenetic marks (green dots) and repressive epigenetic marks (red dots) are indicated. Note that this figure depicts possible, rather than actual, misinterpretations of bulk cell data.

Figure 2. Differences in XCI between human and mouse

Major differences between human and murine XCI have been reported, including imprinted XCI of the Xp in mouse but not human, and possible Xa dampening in human but not mouse. Active X (big arrows), dampened X (small arrows), inactive X (bars), and Xist spreading (green) are indicated in both mouse (blue circles) and human (peach) cells. Zygotic genome activation (ZGA).

Table 1. Currently available single cell genomic techniques

TECHNIQUE	FEATURE DETECTED	REFERENCE	PREVALANCE OF SC PUBLICATIONS
RNA-seq	RNA	[1]	****
BS-Seq	DNA methylation	[10, 11, 12]	***
ChIP-seq	Histone modifications	[13]	*
Hi-C	Chromosome conformation	[14]	*
NOMe-seq	Nucleosome occupancy and DNA methylation	[18]	**
ATAC-seq	DNA accessibility	[15, 16]	**
DNase-seq	DNA accessibility	[17]	**
G&T-seq	Genomic DNA and RNA	[21]	**
DR-seq	Genomic DNA and RNA	[22]	**
M&T-seq	DNA methylation and RNA	[23]	**
NMT-seq	Nucleosome occupancy, DNA methylation and RNA.	[20]	*
COOL-seq	Nucleosome occupancy, DNA methylation, copy number and ploidy	[19]	*
Trio-seq	DNA methylation, Copy number and RNA	[24]	*


a. Heterogeneity
b. Escape
c. Spreading
d. Conformation
e. Mechanism
Single-cell resolution
Bulk resolution
Xa Xa
Xa Xi
Xa Xi
Xa Xi
Heterogeneous XCI
Variable escapees
Heterogeneous spreading of XCI
Xi structure heterogeneous
Correlation implies causality
Xa Xi
Uniform XCI
Constitutive escapees only
No spreading of XCI
No fine Xi structure
Insufficient resolution